Kongeriget Danmark

Patent application No.:   0888/92

Date of filing: 06 Jul 1992

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Int. Cl. 5:   C 12N ·9/20

Title of invention: Lipase variants

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Industriministeriet

Patentdirektoratet

Taastrup 18 Jun 1993

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Assistent

5 LIPASE VARIANTS

FIELD OF THE INVENTION

The present invention relates to novel lipase enzyme variants
10 with improved properties, DNA constructs coding for the ex-
pression of said variants, host cells capable of expressing
the variants from the DNA constructs, as well as a method of
producing the variants by cultivation of said host cells.
Furthermore, the present invention relates to a recombinant
15 essentially pure Candida antarctica lipase and variants
thereof as well as a DNA sequence encoding this lipase.

BACKGROUND OF THE INVENTION

20 A wide variety of lipases of microbial and mammalian origin
are known. The amino acid sequence of many of these lipases
have been elucidated and analyzed with respect to structural
and functional elements important for their catalytic func-
tion, see, for instance, Winkler et al., 1990 and Schrag et
25 al., 1991. It has been found that the lipase enzyme upon
binding of a lipid substrate and activation undergoes a
conformational change, which inter alia, results in an expo-
sure of the active site to the substrate. This conformational
change together with the presumed interaction between enzyme
30 and substrate have been discussed by, inter alia, Brady et
al., 1990, Brzozowski et al., 1991, Derewenda et al., 1992,
and Derewenda and Derewenda, in press.

Based on the knowledge of the structure of a number of lipa-
35 ses, it has been possible to construct lipase variants having
improved properties by use of recombinant DNA techniques.
Thus, WO 92/05249 discloses the construction of certain
lipase variants, in which the lipid contact zone has been
modified so as to provide the variants with different
40 substrate specificities and/or an improved accessibility of

the active site of the lipase to a lipid substrate. The modifications involve changing the electrostatic charge, hydrophobicity or the surface conformation of the lipid contact zone by way of amino acid substitutions.

Although the structural and functional relationship of lipases have been the subject of a number of studies as described in the above cited references, the research has mainly focused on the macroscopic characteristics of the lipases upon substrate binding and activation, whereas the identity of the amino acids actually involved in the substrate binding and catalytic activity has been discussed only to a lesser extent.

SUMMARY OF THE INVENTION

By sequence alignment analysis combined with analysis of the structure and activity of a number of lipases, the present inventors have now surprisingly found that the presence of certain amino acids, especially tryptophan, in a critical position of the lipase seems to be important for optimal catalytic activity.

It is consequently an object of the present invention to modify lipases which do not comprise such an amino acid residue, especially a tryptophan residue, in the critical position (which lipases in the present context are termed parent lipases) by replacing the amino acid residue located in this zone with a tryptophan residue so as to obtain variants having an increased specific activity.

More specifically, in one aspect the present invention relates to a lipase variant of a parent lipase comprising a

trypsin-like catalytic triad including an active serine
located in a predominantly hydrophobic, elongated binding
pocket of the lipase molecule and, located in a critical
position of a lipid contact zone of the lipase structure, an
5   amino acid residue different from a tryptophan residue, which
interacts with a lipid substrate at or during hydrolysis, in
which lipase variant said amino acid residue has been
replaced by a tryptophan residue so as to confer to the
variant an increased specific activity as compared to that of
10   the parent lipase.

In the present context, the term "trypsin-like" is intended
to indicate that the parent lipase comprises a catalytic
triad at the active site corresponding to that of trypsin,
15   i.e. the amino acids Ser, His and one of Asp, Glu, Asn or
Gln.

All lipases studied until now have been found to comprise at
least one surface loop structure (also termed a lid or a
20   flap) which covers the active serine when the lipase is in
inactive form (an example of such a lipase is described by
Brady et al., 1990). When the lipase is activated, the loop
structure is shifted to expose the active site residues,
creating a surface with increased surface hydrophobicity
25   which interacts with the lipid substrate at or during hydrol-
ysis. For the present purpose, this surface is termed the
"lipid contact zone", intended to include amino acid residues
located within or forming part of this surface. These resi-
dues may participate in lipase interaction with the substrate
30   at or during hydrolysis where the lipase hydrolyses
triglycerides from the lipid phase when activated by contact
with the lipid surface. In the present context, the "critical
position" of the lipase molecule is the position in the lipid
contact zone of the lipase molecule, which is occupied by an
35   amino acid residue which interacts with the lipid substrate
and which is different from a tryptophan residue. Further

important parts of the activated lipase molecule are the binding pocket which constitutes the part of the lipid contact zone to which the lipid substrate binds before hydrolysis, and the hydrolysis pocket, in which the hydrolysis of triglycerides takes place. The three-dimensional structure of the lipid contact zone of an activated _Humicola lanuginosa_ lipase molecule is illustrated in Fig. 1B of WO 92/05249.

In another aspect the present invention relates an _C. antarctica_ lipase A which is essentially free from other _C. antarctica_ substances and which comprises the amino acid sequence identified in Fig. 1 or a variant thereof which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of the _C. antarctica_ lipase having the amino acid sequence shown in Fig. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the _C. antarctica_ lipase A.

The _C. antarctica_ lipase A of the invention has a number of desirable properties including a high thermostability and activity at acidic pH and may advantageously be produced by use of recombinant DNA techniques, e.g. using the procedures described below. Thus, the lipase A of the invention may be obtained in a higher purity and a higher amount than the _C. antarctica_ lipase A purified from wild type _C. antarctica_ which is described in WO 88/02775.

Furthermore, the present invention relates to a DNA sequence encoding the _C. antarctica_ lipase A having the amino acid sequence identified in Fig. 1 or a modification of said DNA

sequence encoding a variant of the <u>C. antarctica</u> lipase A as defined above.

In the present context "<u>C. antarctica</u> lipase A" is used
interchangeably with "lipase A" and the variant of the <u>C. antarctica</u> lipase A is termed "lipase A variant".

The present invention also relates to a DNA construct compri-
sing a DNA sequence encoding a lipase variant as indicated
above or a DNA sequence encoding the <u>C. antarctica</u> lipase A,
a recombinant expression vector carrying said DNA construct,
a cell transformed with the DNA construct or the expression
vector, as well as a method of producing a lipase variant of
the invention by culturing said cell under conditions condu-
cive to the production of the lipase variant, after which the
lipase variant is recovered from the culture.

It will be understood that the lipase variants of the present
invention having an increased specific activity as compared
to their parent lipases may be used for the same purposes as
their parent lipases, advantageously in a lower amount due to
their higher specific activity.

Accordingly, the present invention relates to the use of a
lipase variant of the invention as a detergent enzyme; as a
digestive enzyme; in ester hydrolysis, ester synthesis or
interesterification; or the use of the lipase variant to
avoid pitch trouble arising, e.g., in processes for preparing
mechanical pulp and in paper-making processes using mechan-
ical pulp.

DETAILED DISCLOSURE OF THE INVENTION

As indicated above, the present inventors have found that the
presence of certain aromatic amino acids, especially tryp-
tophan, located in the lipid contact zone of the lipase

molecule is important for optimal catalytic activity.

The importance of the presence of the tryptophan residue was
found in connection with a study of mutants of a <u>Humicola</u>

5   <u>lanuginosa</u> lipase which comprises a tryptophan residue at the
critical position in the lipid contact zone, i.e. the amino
acid number 89 in the amino acid sequence of the <u>H.</u>
<u>lanuginosa</u> lipase published in EP 0 305 216. In the <u>H.</u>
<u>lanuginosa</u> mutants this tryptophan residue was replaced by

10   phenylalanine, tyrosine, histidine, isoleucine, glutamic acid
and glycine, respectively. It was found that the specific
activity of these mutants decreased (in the order indicated
above) from 100% of the wild type lipase to about 10% of the
phenylalanine mutant and down to about 2% for the glycine

15   mutant.

While the critical position in some lipases is contemplated
to be any position within the lipid contact zone, the criti-
cal position will normally be located in the binding pocket

20   of the lipase molecule, and preferably in the hydrolysis
pocket thereof. For most lipases it is believed that the
critical amino acid residue is positioned on top of or in the
proximity of the active site.

25   The amino acid residue occupying this position may be identi-
fied in any lipase by 1) sequence alignment studies in which
the amino acid sequence of the lipase in question is aligned
with the amino acid sequence of other lipases, in which the
amino acid residue positioned on top of or in the proximity

30   of the active serine has been identified, so as to identify
the presumed position of said amino acid residue, and/or 2)
an analysis of the three-dimensional structure of the lipase
in question using standard display programmes such as INSIDE
so as identify the amino acid sequence on top of or in the

35   proximity of the active serine.

In some lipases the critical amino acid residue is located in
the surface loop structure or in one or more of the surface
loop structures if the lipase comprises more than one surface
loop structure.

The amino acid residue present in the critical position, e.g.
on top of or in the proximity of the active serine, is
believed to be involved in a) stabilization of the
tetrahedral intermediate formed from the lipase and the
substrate during the activation of the lipase, and b) in the
activation of the replacement of the lid region covering the
active serine in the inactive enzyme. When tryptophan is
present in this position, it is contemplated that optimal
performance with respect to a) as well as b) above is
obtained. Thus, it is believed that tryptophan gives rise to
the formation of the most stable tetrahedral intermediate
(which means a lowering of the activation energy needed for
the catalysis to take place), and further improves the per-
formance of the enzyme with respect to the activation of the
lid opening which is essential for any catalysis to take
place.

In connection with a) above it has been observed that the
best acting lipase variants contain an unsaturated ring
system in the side-chain. The far the biggest unsaturated
system is tryptophan, then tyrosine, phenylalanine and histi-
dine. These sidechains have a pi-electron system ("the unsa-
turation") that could be important for the proton transfer in
the catalysis resulting in a lower activation energy for
creating the tetrahedral intermediate where proton transfer
has taken place from active site histidine to serine to the
oxyanion hole created after lid activation and opening.

From the above theoretical explanation it will be understood
that the optimal amino acid to be present in the critical
position, e.g. on top of or in the proximity of the active

serine, is tryptophan. However, when the parent lipase is one
which does not contain any aromatic amino acid residue or any
amino acid residue with an unsaturated ring system in the
side-chain in this position, such amino acids may advantage-
5   ously be substituted into this position.

Thus, when the parent lipase, in the critical position, has
an amino acid residue which does not comprise an unsaturated
ring system in the side-chain, an amino acid residue having
10   such an unsaturated ring-system, e.g. an aromatic amino acid
(tryptophan, tyrosine or phenylalanine) or histidine may be
substituted into the critical position. When the amino acid
residue in the critical position of the parent lipase is
histidine, it may advantageously be replaced by
15   phenylalanine, tyrosine and most preferably tryptophan, when
the amino acid residue is tyrosine, it may advantageously be
replaced by phenylalanine and most preferably tryptophan, and
when the amino acid residue is phenylalanine it may advan-
tageously be replaced by tryptophan.
20

Although the critical position is normally considered to be
constituted of only one amino acid residue it may be advan-
tageous to replace two or more residues, preferably with a
tryptophan residue as explained above, in order to obtain a
25   further increased specific acitivity.

It is contemplated that it is possible to increase the speci-
fic activity of parent lipases which do not have a tryptophan
residue in the critical position at least 2 times, such as at
30   least 3 and preferably at least 4 or even 5, 6 or 7 times by
modifications as diclosed herein.

It is contemplated that lipase variants as defined herein
having an increased substrate specificity may be prepared on
35   the basis of parent lipases of various origins. Thus, the
parent lipase may be a microbial lipase or a mammalian lipa-

se.

When the parent lipase is a microbial lipase, it may be
selected from yeast, e.g. Candida, lipases, bacterial, e.g.
5   Pseudomonas, lipases or fungal, e.g. Humicola or Rhizomucor
lipases.

One preferred lipase variant is one, in which the parent
lipase is derived from a strain of Candida antarctica, in
10   particular one in which the parent lipase is lipase A of C.
antarctica, preferably the one which has the amino acid
sequence shown in Fig. 1 or a lipase A variant thereof as
defined herein. The lipase variant of this C. antarctica
lipase A preferably has the amino acid sequence shown in Fig.
15   1 in which the phenylalanine 139 of the parent lipase has
been replaced by a tryptophan residue. The construction of
this variant and the analysis of the properties thereof is
discussed in Example 3, 5 and 6.

20   A lipase variant of the invention may, as mentioned above, be
prepared on the basis of a parent lipase derived from a
strain of a Pseudomonas species, e.g. Ps. fragi. An example
of a suitable Ps. fragi lipase which has an amino acid resi-
due different from tryptophan positioned on top of or in the
25   proximity of the active serine, is the one described by
Aoyama et al., 1988. A lipase variant according to the
present invention may be constructed by replacing the
phenylalanine residue 29 in the amino acid sequence of said
lipase shown in Fig. 2 by a tryptophan residue.

30

An example of a fungal lipase suitable as a parent lipase for
the construction of a lipase variant of the invention is one
derived from Rhizopus, especially from R. delemar or R.
niveus, the amino acid sequence of which latter is disclosed
35   in, e.g., JP 64-80290. In order to construct a lipase variant
according to the present invention from this parent lipase,

the alanine residue at position 117 is to be replaced with a
tryptophan residue. The sequence alignment of the R. niveus
lipase sequence and an Rhizomucor miehei lipase sequence
(containing a tryptophan residue) is illustrated in Fig. 3.

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The present inventors have surprisingly found that non-pan-
creatic lipases such as gastric, lingual, or hepatic lipases
have the common feature that the amino acid residue which has
been identified to be the one located in the critical posi-
10   tion of the lipase molecule, normally on top of or in the
proximity of the active serine, is different from tryptophan.
This is in contrast to pancreatic lipases which generally
have been found to have a tryptophan residue in this posi-
tion. Thus, in the present context, non-pancreatic mammalian
15   lipases may advantageously be used as "parent lipases" for
the construction of lipase variants of the invention.

Accordingly, lipase variants as disclosed herein which is of
mammalian origin is advantageously prepared from a parent
20   lipase of non-pancreatic, such as gastric, lingual or hepatic
origin. Such mammalian lipases may be derived from humans,
rats, mice, pigs, dogs or other mammals. Specific examples of
such mammalian lipases includes a rat lingual lipase (Docher-
ty et al., 1985), a rat hepatic lipase (Komaromy and Schotz,
25   1987), a human hepatic lipase (Datta et al., 1988), a human
gastric lipase (Bodmer et al., 1987), and a Bio Salt Acti-
vated Lipase (BSAL) (Baba et al., 1991) all of which were
analysed with respect to the critical position in the
sequence alignment analysis illustrated in Fig. 4. The pan-
30   creatic lipases included in this sequence alignment study
were a murine pancreatic lipase (Grusby et al., 1990), a
porcine pancreatic lipase (Caro et al., 1981), a human pan-
creatic lipase (Lowe et al., 1989), and a canine pancreatic
lipase (Mickel et al., 1989).

35

As mentioned above the present invention also relates to a C.

antarctica lipase A essentially free from other C. antarctica
substances, which has the amino acid sequence shown in Fig. 1
or a variant therof which

5   1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope
of C. antarctica lipase A having the amino acid sequence
shown in Fig. 1, and/or

10

3) is encoded by a nucleotide sequence which hybridizes with
an oligonucleotide probe prepared on the basis of the full or
partial nucleotide sequence shown in Fig. 1 encoding the C.
antarctica lipase A.

15

In the present context, the term "variant" is intended to
indicate a lipase A variant which is derived from the C.
antarctica lipase A having the amino acid sequence shown in
Fig. 1, or a naturally occurring variant. Typically, the

20   variant differ from the native lipase A by one or more amino
acid residues, which may have been added or deleted from
either or both of the N-terminal or C-terminal end of the
lipase, inserted or deleted at one or more sites within the
amino acid sequence of the lipase or substituted with one or

25   more amino acid residues within, or at either or both ends of
the amino acid sequence of the lipase.

Furthermore, the variant of the invention has one or more of
the characterizing properties 1)-3) mentioned above. Property

30   1), i.e. the "lipase activity" of the variant may be deter-
mined using any known lipase assay, e.g. the Standard LU
assay described in the Methods section below.

Property 2), i.e. the reactivity of the variant of the inven-

35   tion with an antibody raised against or reactive with at
least one epitope of the C. antarctica lipase A having the

amino acid sequence shown in Fig. 1 below may be determined by polyclonal antibodies produced in a known manner, for instance by immunization of a rabbit with the C. antarctica lipase A of the invention. The antibody reactivity may be determined using assays known in the art, examples of which are Western Blotting or radial immunodiffusion assay.

Property 3) above, involving hybridization, may be performed using an oligonucleotide probe prepared on the basis of the full or partial cDNA sequence encoding the C. antarctica lipase A, the amino acid sequence of which is identified in Fig. 1, as a hybridization probe in a hybridization experiment carried out under standard hybridization conditions, e.g. 6 X SSC at 50°C. The nucleotide sequence on the basis of which the oligonucleotide probe is prepared is conveniently the DNA sequence shown in Fig. 1.

As stated above in a further aspect the present invention relates to a DNA sequence encoding C. antarctica lipase A having the amino acid sequence shown in Fig. 1 or a modification of said DNA sequence which encodes a variant of C. antarctica lipase A which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of the C. antarctica lipase A having the amino acid sequence shown in Fig. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the C. antarctica lipase A.

Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another

amino acid sequence of the encoded enzyme, but which may
correspond to the codon usage of the host organism into which
the DNA sequence is introduced or nucleotide substitutions
which do give rise to a different amino acid sequence, with-

5   out, however, impairing the above stated properties of the
enzyme. Other examples of possible modifications are inser-
tion of one or more nucleotides into the sequence, addition
of one or more nucleotides at either end of the sequence and
deletion of one or more nucleotides at either end of or

10 within the sequence.

Methods of preparing lipase variants of the invention

Several methods for introducing mutations into genes are

15 known in the art. After a brief discussion of cloning lipase-
encoding DNA sequences, methods for generating mutations at
specific sites within the lipase-encoding sequence will be
discussed.

20 ## Cloning a DNA sequence encoding a lipase

The DNA sequence encoding a parent lipase or the C.
antarctica lipase A may be isolated from any cell or micro-
organism producing the lipase in question by various methods,

25 well known in the art. First a genomic DNA and/or cDNA
library should be constructed using chromosomal DNA or
messenger RNA from the organism that produces the lipase to
be studied. Then, if the amino acid sequence of the lipase is
known, homologous, labelled oligonucleotide probes may be

30 synthesized and used to identify lipase-encoding clones from
a genomic library of bacterial DNA, or from a fungal cDNA
library. Alternatively, a labelled oligonucleotide probe
containing sequences homologous to lipase from another strain
of bacteria or fungus could be used as a probe to identify

35 lipase-encoding clones, using hybridization and washing
conditions of lower stringency.

Yet another method for identifying lipase-producing clones
would involve inserting fragments of genomic DNA into an
expression vector, such as a plasmid, transforming lipase-
negative bacteria with the resulting genomic DNA library, and
5   then plating the transformed bacteria onto agar containing a
substrate for lipase. Those bacteria containing lipase-be-
aring plasmid will produce colonies surrounded by a halo of
clear agar, due to digestion of the substrate by secreted
lipase.

10

Alternatively, the DNA sequence encoding the enzyme may be
prepared synthetically by established standard methods, e.g.
the phosphoamidite method described by S.L. Beaucage and M.H.
Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or
15   the method described by Matthes et al., The EMBO J. 3, 1984,
pp. 801-805. According to the phosphoamidite method, oligo-
nucleotides are synthesized, e.g. in an automatic DNA synthe-
sizer, purified, annealed, ligated and cloned in appropriate
vectors.

20

Finally, the DNA sequence may be of mixed genomic and synthe-
tic, mixed synthetic and cDNA or mixed genomic and cDNA
origin prepared by ligating fragments of synthetic, genomic
or cDNA origin (as appropriate), the fragments corresponding
25   to various parts of the entire DNA sequence, in accordance
with standard techniques. The DNA sequence may also be pre-
pared by polymerase chain reaction (PCR) using specific
primers, for instance as described in US 4,683,202 or R.K.
Saiki et al., Science 239, 1988, pp. 487-491.

30

Site-directed mutagenesis of the lipase-encoding sequence

Once a lipase-encoding DNA sequence has been isolated, and
desirable sites for mutation identified, mutations may be
35   introduced using synthetic oligonucleotides. These oligo-
nucleotides contain nucleotide sequences flanking the desired

mutation sites; mutant nucleotides are inserted during oligo-
nucleotide synthesis. In a specific method, a single-stranded
gap of DNA, bridging the lipase-encoding sequence, is created
in a vector carrying the lipase gene. Then the synthetic

5   nucleotide, bearing the desired mutation, is annealed to a
homologous portion of the single-stranded DNA. The remaining
gap is then filled in with DNA polymerase I (Klenow fragment)
and the construct is ligated using T4 ligase. A specific
example of this method is described in Morinaga et al.,

10   (1984, Biotechnology 2:646-639). U.S. Patent number
4,760,025, by Estell et al., issued July 26, 1988, discloses
the introduction of oligonucleotides encoding multiple muta-
tions by performing minor alterations of the cassette, howe-
ver, an even greater variety of mutations can be introduced

15   at any one time by the Morinaga method, because a multitude
of oligonucleotides, of various lengths, can be introduced.

Another method of introducing mutations into lipase-encoding
sequences is described in Nelson and Long, Analytical Bi-
20   ochemistry 180, 1989, pp. 147-151. It involves the 3-step
generation of a PCR fragment containing the desired mutation
introduced by using a chemically synthesized DNA strand as
one of the primers in the PCR reactions. From the PCR-ge-
nerated fragment, a DNA fragment carrying the mutation may be

25   isolated by cleavage with restriction endonucleases and
reinserted into an expression plasmid.

Expression of lipase variants
According to the invention, a C. antarctica lipase A-coding
30   sequence or a mutated lipase-coding sequence produced by
methods described above, or any alternative methods known in
the art, can be expressed, in enzyme form, using an
expression vector which typically includes control
sequences encoding a promoter, operator, ribosome binding
35   site, translation initiation signal, and, optionally, a
repressor gene or various activator genes. To permit the

secretion of the expressed protein, nucleotides encoding a "signal sequence" may be inserted prior to the lipase-coding sequence. For expression under the direction of control sequences, a target gene to be treated according to the

5　　invention is operably linked to the control sequences in the proper reading frame. Promoter sequences that can be incorporated into plasmid vectors, and which can support the transcription of the mutant lipase gene, include but are not limited to the prokaryotic ß-lactamase promoter (Villa-Ka-

10　　maroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

15

According to one embodiment B. subtilis is transformed by an expression vector carrying the lipase A or the mutated DNA. If expression is to take place in a secreting microorganism such as B. subtilis a signal sequence may follow the trans-

20　　lation initiation signal and precede the DNA sequence of interest. The signal sequence acts to transport the expression product to the cell wall where it is cleaved from the product upon secretion. The term "control sequences" as defined above is intended to include a signal sequence, when

25　　is present.

In a currently preferred method of producing lipase A or lipase variants of the invention, a filamentous fungus is used as the host organism. The filamentous fungus host

30　　organism may conveniently be one which has previously been used as a host for producing recombinant proteins, e.g. a strain of Aspergillus sp., such as A. niger, A. nidulans or A. oryzae. The use of A. oryzae in the production of recombinant proteins is extensively described in, e.g. EP 238 023.

35

For expression of lipase variants in Aspergillus, the DNA

sequence coding for the lipase A or the lipase variant is preceded by a promoter. The promoter may be any DNA sequence exhibiting a strong transcriptional activity in <u>Aspergillus</u> and may be derived from a gene encoding an extracellular or

5   intracellular protein such as an amylase, a glucoamylase, a protease, a lipase, a cellulase or a glycolytic enzyme.

Examples of suitable promoters are those derived from the gene encoding <u>A. oryzae</u> TAKA amylase, <u>Rhizomucor miehei</u>

10   aspartic proteinase, <u>A. niger</u> neutral α-amylase, <u>A. niger</u> acid stable α-amylase, <u>A. niger</u> glucoamylase, <u>Rhizomucor miehei</u> lipase, <u>A. oryzae</u> alkaline protease or <u>A. oryzae</u> triose phosphate isomerase.

15   In particular when the host organism is <u>A. oryzae</u>, a preferred promoter for use in the process of the present invention is the <u>A. oryzae</u> TAKA amylase promoter as it exhibits a strong transcriptional activity in <u>A. oryzae</u>. The sequence of the TAKA amylase promoter appears from EP 238 023.

20

Termination and polyadenylation sequences may suitably be derived from the same sources as the promoter.

The techniques used to transform a fungal host cell may

25   suitably be as described in EP 238 023.

To ensure secretion of the lipase A or the lipase variant from the host cell, the DNA sequence encoding the lipase variant may be preceded by a signal sequence which may be a

30   naturally occurring signal sequence or a functional part thereof or a synthetic sequence providing secretion of the protein from the cell. In particular, the signal sequence may be derived from a gene encoding an <u>Aspergillus</u> sp. amylase or glucoamylase, a gene encoding a <u>Rhizomucor miehei</u> lipase or

35   protease, or a gene encoding a <u>Humicola</u> cellulase, xylanase or lipase. The signal sequence is preferably derived from the

gene encoding A. oryzae TAKA amylase, A. niger neutral α-amylase, A. niger acid-stable α-amylase or A. niger glucoamylase.

5   The medium used to culture the transformed host cells may be any conventional medium suitable for culturing Aspergillus cells. The transformants are usually stable and may be cultured in the absence of selection pressure. However, if the transformants are found to be unstable, a selection marker

10   introduced into the cells may be used for selection.

The mature lipase protein secreted from the host cells may conveniently be recovered from the culture medium by well-known procedures including separating the cells from the

15   medium by centrifugation or filtration, and precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

20

It will be understood that the lipase variants of the invention are contemplated to be active towards the same type of substrates as their parent lipases, with an improved specific activity. Thus, the lipase variants of the invention

25   are contemplated to be useful for the same purposes as their parent lipases.

Accordingly, lipase variants of the invention prepared from a parent lipase useful as a detergent enzyme may be used as an

30   active ingredient in a detergent additive or a detergent composition.

Another contemplated use of lipase variants of the invention, is as digestive enzymes, e.g. in the treatment of cystic

35   fibrosis.

A third use of the lipase variants of the invention, especially variants of C. antarctica lipases are in lipase-catalysed processes such as in ester hydrolysis, ester synthesis and interesterification. The use of lipases in these pro-
5   cesses is discussed in detail in WO 88/02775 (Novo Nordisk A/S), the content of which is incorporated herein by reference. Also the lipase variants of the invention may be used to avoid pitch trouble that arises in the production process for mechanical pulp or in a paper-making process using mech-
10   anical pulp, e.g. as described in PCT/DK92/00025 (Novo Nordisk A/S), the content of which is incorporated herein by reference.

     BRIEF DESCRIPTION OF THE DRAWINGS

15

The present invention is described in the following with reference to the appended drawings, in which

Fig. 1A illustrates the amino acid sequence of the mature C.
20   antarctica lipase A,

Fig. 1b illustrates the amino acid sequence of the C. antarctica lipase A preproenzyme,

25   Fig. 1C illustrates the amino acid and nucleotide sequence of the C. antarctica lipase A of the invention,

Fig. 2 is the amino acid sequence of the Ps. fragi lipase described by Aoyama et al. 1988,

30

Fig. 3 is an amino acid sequence alignment of a R. niveus lipase sequence and an Rhizomucor miehei lipase sequence from which the critical position of the R. niveus lipase may be determined,

35

Fig. 4 illustrates the sequence alignment of a number of

mammalian pancreatic and non-pancreatic lipases, in which

1) is the amino acid sequence of the Bio Salt Activated
Lipase described by Baba et al., 1991,

2) is the amino acid sequence of the rat ligual lipase
described by Docherty et al., 1985,

3) is the amino acid sequence of the human gastric lipase
described by Bodmer et al., 1987,

4) is the amino acid sequence of the dog pancreatic lipase
described by Mickel et al., 1989,

5) is the amino acid sequence of the human pancreatic lipase
described by Lowe et al., 1989,

6) is the amino acid sequence of the murine pancreatic lipase
described by Grusby et al., 1990,

7) is the amino acid sequence of the porcine pancreatic
lipase described by Caro et al., 1981,

8) is the amino acid sequence of the human hepatic lipase
described by Datta et al., 1988, and

9) is the amino acid sequence of the rat hepatic lipase
described by Komaromy et al., 1987,

Fig. 5 illustrates the N-terminal sequence of the lipase A
from the _C. antarctica_ strain LF 058, and the nucleotide
sequences of the oligonucleotide probes NOR 438 and NOR 440
(see Example 1),

Fig. 6 illustrates the nucleotide sequence covering the open
reading frame of the _C. antarctica_ lipase A of the invention

(see Example 1),

Fig. 7 is a table showing the amino acid compositions of the
C. antarctica lipase A of the invention and of a purfied wild
5   type lipase A, and

Figs. 8 and 9 illustrate the scheme for the construction of
the expression plasmid pMT1229 (see Example 1).

10   The present invention is further illustrated in the following
examples which are not intended, in any way, to limit the
scope of the invention as claimed.

GENERAL METHODS

15

Site-directed in vitro mutagenesis of lipase genes

The three different approaches described in WO 92/05249 may
be used for introducing mutations into the lipase genes, i.e.
20   the oligonucleotide site-directed mutagenesis which is desc-
ribed by Zoller & Smith, DNA, Vol. 3, No. 6, 479-488 (1984),
the PCR method as described in Nelson & Long, Analytical
Biochemistry, 180, 147-151 (1989), and the so-called
"cassette mutagenesis" technique, in which a segment between
25   two restriction sites of the lipase-encoding region is
replaced by a synthetic DNA fragment carrying the desired
mutation. Use of the latter technique is illustrated in
Example 2.

30   Determination of lipase specific activity

Lipase activity was assayed using glycerine tributyrat as a
substrate and gum-arabic as an emulsifier. 1 LU (Lipase Unit)
is the amount of enzyme which liberates 1 μmol titratable
35   butyric acid per minute at 30°C, pH 7.0. The lipase activity
was assayed by pH-stat using Radiometer titrator VIT90,

Radiometer, Copenhagen. Further details of the assay are given in Novo Analytical Method AF 95/5, available on request.

5   EXAMPLE 1

Cloning of Candida antarctica lipase A

Chromosomal DNA of the C. antarctica strain LF058 was
10   prepared by opening of frozen cells by grinding with quartz
and subsequent extraction of DNA essentially as described by
Yelton et al., (1984). The purified DNA was cut partially
with Sau3A and, after agarose gel electrophoresis, fragments
in the range of 3-9 kb were isolated. The sized Sau3A
15   fragments were ligated into a BamH1-cut, dephosphorylated
plasmid pBR322 (New England Biolabs). The ligation mix was
transformed into the K12 restriction deficient E. coli MC1000
derivative MT172. Approximately 50,000 transformant E. coli
colonies were obtained, 80% of which contained an insert of
20   LF058 DNA.

Using standard colony hybridization techniques (Maniatis et
al., 1982) the colonies were screened with the 32^P-phosphory-
lated oligonucleotide probe NOR 440. NOR 440 is a degenerated
25   (64) 17 mer based on the N-terminal determined from mature C.
antarctica lipase A (see Fig. 1). 34 colonies appeared posi-
tive after wash at low stringency (41°C and 6 x SSC). Plasm-
ids were prepared from these colonies and Southern analyzed
after restriction with BstN1. The probe for the Southern was
30   either the NOR 440 probe used for the colony hybridization
(see above) or a 32^P-labelled probe NOR 438. NOR 438 is an
oligonucleotide (a guess mer) where, at 13 positions, a base
has been chosen on the basis of codon use in yeasts and
filamentous fungi. Only one plasmid, pMT1076, contained a
35   band which hybridised both to NOR 440 at low stringency (see
above) and to NOR 438 at a somewhat higher stringency (55°C

and 1 x SSC).

PMT1076 was restriction mapped and the DNA sequence determined by the Maxam-Gilbert method. The sequence covering the

5   open reading frame is shown in Fig. 6. The open reading frame is seen to encode a putative signal sequence of 21 amino acids (according to the von Heine rules (von Heijne, G. (1986)) and furthermore a propeptide of 10 amino acids preceding the N-terminal of the mature lipase. The last two

10 amino acids of the propeptide are Arg Arg, i.e. a typical cleavage site for endoproteolytic processing by enzymes of the S. cereviciae KEX-2 type. The amino acid composition of the mature protein encoded by the DNA sequence is in agreement with the amino acid composition determined for C. ant-

15 arctica lipase A (Fig. 7).

Through a number of standard plasmid manipulations (Maniatis et al., 1982) illustrated in Figs. 8 and 9, the open reading frame of C. antarctica lipase A was placed in the correct

20 orientation between the alpha-amylase promoter of A. oryzae and the glucoamylase transcription terminator of A. niger. The resulting expression plasmid pMT1229 was transformed into A. oryzae A1560 as described in EP 305,216. Transformants were isolated and grown as described in the above cited

25 patents and the culture supernatants were analyzed for the presence of C. antarctica lipase A.

EXAMPLE 2

30 **Construction of a plasmid expressing the F135W variant of Candida antarctica lipase A**

A 246 bp BamHI/BssHII fragment was synthesized in vitro on the basis of the nucleotide sequence of pMT1229 using oligo-

35 nucleotide primers 3116 and 3117 in a PCR reaction. The primer 3117 includes a BssHII restriction site and a mutation

in the 135 phe codon (TTC) to trp codon (TGG) which is marked
with stars.

5 Oligonucleotide primer 3116 (F135W:710-730)
   5'-CAG AAC GAG GCG GTG GCC GAC-3'

   Oligonucleotide primer 3117 (F135W:1020-940)
   5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT
10 TCG TAG CCA GCG ATG AAG GCG GCT TTC* C*AG CCT TCG TG-3'

The PCR reaction was performed by mixing the following com-
ponents and incubating the mixture in a HYBAID™ thermal
reactor.

15
Template pMT1229	10 ng/μl	1 μl
H$_2$O		46.5μl
10 x PCR buffer		10 μl
2 mM dATP		10 μl
2 mM dTTP		10 μl
2 mM dCTP		10 μl
2 mM dGTP		10 μl
primer 3116	50.5 pmol/μl	1 μl
primer 3117	70.5 pmol/μl	1 μl
Taq polymerase		0.5μl
Parafin oil		50 μl

20

25

Step I	94°C	2 min.	1 cycle
Step II	94°C	30 sec.	
	50°C	30 sec.	30 cycle
	72°C	2 min.	
Step III	72°C	5 min.	1 cycle

30

35

The resulting 310 bp fragment was isolated from a 2% agarose
gel after electrophoresis and digested with BamHI and BssHII
restriction enzymes. The resulting 264 bp BamHI/BssHII frag-
40 ment was likewise isolated from 2% agarose gel. This fragment
   was then ligated with pMT1229   BamHI/XbaI 0.3 kb
                          pMT1229   BssHII/SphI 0.5 kb

pMT1229   SphI/XbaI   5.0 kb

The ligated DNA was transformed into E. coli strain MT172.
Transformants which contained correct inserts were selected
5   and their DNA sequence was determined by use of Sequenase
(United States Biochemical Corporation). The pME1178 plasmid
contained a mutation in the amino acid position 135 (phe was
mutated to trp).

10   pME1178 was cotransformed with pToC186 which included the
amdS gene from A. nidulans as a selective marker into the
Aspergillus oryzae A1560 strain. A. oryzae transformants were
reisolated twice on selective plates and stable transformants
were characterized by rocket immunoelectrophoresis, using
15   anti-Candida lipase A antibody. Candida lipase A produced by
the MEA65 strain was further analyzed for specific activity.

EXAMPLE 3
20

Construction of a plasmid expressing the F139W variant of
Candida antarctica lipase A

A 246 bp BamHI/BssHII fragment was synthesized in vitro on
25   the basis of the nucleotide sequence of the plasmid pMT1229
using oligonucleotide primers 3116 and 3826 in a PCR reac-
tion. The primer 3826 includes a BssHII restriction site and
a mutation in the 139 phe codon (TTC) to trp codon (TGG)
which is marked with stars.
30
Oligonucleotide primer 3116 is shown in Example 2.

Oligonucleotide primer 3826 (F139W:1020-941)
5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT
35   TCG TAG CCA GCG ATC* C*AG GCG GCT TTG AAG CCT TCG TG-3'

A PCR reaction was performed by the method described in
Example 2. The 310 bp fragment was isolated from 2% agarose
gel after electrophoresis and digested by BamHI and BssHII
restriction enzymes. The resulting 264 bp BamHI/BssHII

5   fragment was likewise isolated from 2% agarose gel. This
fragment was then ligated with   pMT1229 BamHI/XbaI   0.3 kb
                                   pMT1229 BssHII/SphI 0.5 kb
                                   pMT1229 SphI/XbaI   5.0 kb

10   The ligated DNA was transformed into E. coli strain MT172.
Transformants which contained correct inserts were selected
and their DNA sequence was determined by use of Sequenase
(United States Biochemical Corporation). The pME1229 plasmid
contained a mutation in the amino acid position 139 (phe was

15   mutated to trp).

pME1229 was cotransformed with pToC202 which included the
amdS gene from A. nidulans as a selective marker into Asper-
gillus oryzae A 1560 strain. A oryzae transformants were

20   reisolated twice on selective plates and enzyme activity of a
stable transformant MEA155 was analyzed by using tributylene
as a substrate as described in Example 5.

EXAMPLE 4

25

**Construction of a plasmid expressing the F135W/F139W variant
of Candida antarctica lipase A**

A 246 bp BamHI/BssHII fragment was synthesized in vitro using

30   oligonucleotide primers 3116 and 4224 by a PCR reaction. The
primer 4224 includes a BssHII restriction site and mutations
in the 135 and 139 codons (TTC) to trp codons (TGG) which are
marked with stars.

35   The oligonucleotide primer 3116 is shown in Example 2.

Oligonucleotide primer 4224 (F135W:1020-941)
5'-TTC TTG AGC GCG CGG ATG CCG TCG AGG ATA GCC ATG CCC TCT
TCG TAG CCA GCG ATC* C*AG GCG GCT TTC* C*AG CCT TCG TG-3'

5   PCR reaction was performed by using the method shown in
Example 2. The 310 bp fragment was isolated from a 2% agarose
gel after electrophoresis and digested with BamHI and BssHII
restriction enzymes. The resulting 264 bp BamHI/BssHII
fragment was likewise isolated from a 2% agarose gel. This
10   fragment was then ligated with   pMT1229   BamHI/XbaI   0.3 kb
                                        pMT1229   BssHII/SphI 0.5 kb
                                        pMT1229   SphI/XbaI   5.0 kb

The ligated DNA was transformed into E. coli MT172. Transfor-
15   mants which contained inserts were selected and their DNA
sequence was determined by use of Sequenase. The pME1230
plasmid contained two mutations in the amino acid positions
135 and 139 (phe was mutated to trp).

20   pME1230 was cotransformed with pToC90 which included the amdS
gene from A. nidulans as a selective marker into Aspergillus
oryzae A 1560 strain. A. oryzae transformants were reisolated
twice on selective plates and enzyme activity of stable
transformants were analyzed by using tributylene as a
25   substrate as described in Example 5.

EXAMPLE 5

30

Purification of C. antarctica lipase A variants F139W and
F135W/F139W and comparison of specific activity with their
parent wild type C. antarctica lipase A

35   The lipase variants and the parent lipase produced as descri-
bed in Examples 3, 4 and 1, respectively, were purified using

the following 4 step standard purification procedure.

Step 1: The fermentation broth containing the lipase and lipase variant, respectively, obtained by culturing the
5   transformed A. oryzae cells described in Examples 1 and 3 above, was centrifuged, and the supernatant was adjusted to pH 7. Ionic strength was adjusted to 2 mSi. DEAE-Sephadex A-50 (Pharmacia) gel was swollen and equilibrated in 25 mM Tris acetate buffer pH 7. The fermentation supernatant was passed
10   through DEAE-Sephadex A-50 on scintered glass funnel. The effluent containing lipase activity was collected and adjusted to 0.8 M ammonium acetate.

Step 2: An appropriate column was packed with TSK gel Butyl-
15   Toyopearl 650 C and equilibrated with 0.8 M ammonium acetate. The effluent containing lipase activity was applied on the column. The bound material was eluted with water.

Step 3: The lipase-containing eluate was then applied on a
20   Highperformance Q-Sepharose column. Lipase activity was collected as effluent. The lipases purified by this method were concentrated to an Optical Density of 1 at 280 nm.

The purity of the lipases was checked by SDS-PAGE showing one
25   band with an molecular weight of about 45 kD. The lipase activity was determined by use of the method outlined above in the section "General methods".

The lipase activity of the parent wild type lipase was 300
30   LU/OD_{280} as compared to 1200 LU/OD_{280} for the lipase variant F139W. On the basis of OD_{280} absorption without correction for the inserted tryptophan, the specific activity of the mutant was at least four times higher with the assay used. The lipase activity of the lipase variant F135W/F139W was 1400
35   LU/OD_{280} (without correction for the two additional tryptophans).

EXAMPLE 6

Thermostability of <u>Candida antarctica</u> lipase A and the mutant F139W thereof

5

The thermostability of the <u>C. antarctica</u> lipase A and the <u>C. antarctica</u> lipase A variant, was examined by Differential Scanning Calorimetry (DSC) at different pH values. Using this technique, the thermal denaturation temperature, T_d, is deter-

10 mined by heating an enzyme solution at a constant programmed rate.

More specifically, the Differential Scanning Calorimeter, MC-2D, from MicroCal Inc. was used for the investigations.

15 Enzyme solutions were prepared in 50 mM buffer solutions, cf. the tables below. The enzyme concentration ranged between 0.6 and 0.9 mg/ml, and a total volume of about 1.2 ml was used for each experiment. All samples were heated from 25°C to 90°C at a scan rate of 90°C/hr.

20

The results obtained from the analysis is shown in the table below:

C. ant. lipase A (WT)

pH	Buffer (50 mM)	Denaturation temperature[1]
4.5	Acetate	96°C
5	Acetate	95°C
7	TRIS	93°C

C. ant. lipase A mutant (F139W)

pH	Buffer (50 mM)	Denaturation temperature[1]
5	Acetate	84°C
7	TRIS	82°C

[1] Temperature, at which approximately half the enzyme molecules present have been denatured thermally during heating

The above results show that the pH-optimum for the thermostability of C. antarctica lipase A and the F139W variant is unusually low and that both enzymes are very thermostable below pH 7. Within the investigated range the thermostability of both the Wild Type and the mutant F139W continues to

increase as pH is lowered. This makes both lipases very well suited for hydrolysis/synthesis at unusually high temperatures at relatively low pH values.

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CLAIMS

1. A lipase variant of a parent lipase comprising a trypsin-
like catalytic triad including an active serine located in a
predominantly hydrophobic, elongated binding pocket of the
lipase molecule and, located in a critical position of a
lipid contact zone of the lipase structure, an amino acid
residue different from a tryptophan residue, which interacts
with a lipid substrate at or during hydrolysis, in which
lipase variant said amino acid residue has been replaced by a
tryptophan residue so as to confer to the variant an increa-
sed specific activity as compared to that of the parent
lipase.

2. A lipase variant according to claim 1, in which said amino
acid residue different from a tryptophan residue is located
in the binding pocket of the lipase molecule.

3. A lipase variant according to claim 2, in which said amino
acid residue different from a tryptophan residue is located
in a hydrolysis pocket of the lipase molecule.

4. A lipase variant according to any of claims 1-3, wherein
said amino acid residue different from a tryptophan residue
is located on top of or in the proximity of the active seri-
ne.

5. A lipase variant according to any of claims 1-4, wherein
said amino acid residue different from a tryptophan residue
is present in a lid region of the lipase.

6. A lipase variant according to any of the preceding claims,
in which said amino acid residue different from a tryptophan
residue is a phenylalanine residue.

7. A lipase variant according to any of the preceding claims, wherein the parent lipase is selected from a microbial or a mammalian lipase.

5 8. A lipase variant according to claim 7, wherein the parent lipase is a yeast lipase.

9. A lipase variant according to claim 8, wherein the parent lipase is derived from a strain of <u>Candida antarctica</u>.

10

10. A lipase variant according to claim 9, wherein the parent lipase is lipase A of <u>C. antarctica</u>.

11. A lipase variant according to claim 7, which has the

15 amino acid sequence shown in Fig. 1 in which the phenylalanine 139 of the parent lipase has been replaced by a tryptophan residue.

12. A lipase variant according to claim 4, in which the

20 parent lipase is a bacterial lipase.

13. A lipase variant according to claim 12, wherein the parent lipase is derived from a strain of <u>Pseudomonas</u>.

25 14. A lipase variant according to claim 13, which is derived from a strain of <u>Ps. fragi</u>.

15. A lipase variant according to claim 14, which has the amino acid sequence shown in Fig. 2 in which the

30 phenylalanine 29 of the parent lipase has been replaced by a tryptophan residue.

16. A lipase variant according to claim 7, wherein the parent lipase is a fungal lipase.

35

17. A lipase variant according to claim 7, wherein the parent

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lipase is a human lipase, a murine lipase, a rat lipase or a canine lipase.

18. A <u>C. antarctica</u> lipase A essentially free from other substances from <u>C. antarctica</u>, which comprises the amino acid sequence shown in Fig. 1, or a variant of said lipase which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of <u>C. antarctica</u> lipase A having the amino acid sequence SEQ ID No. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the <u>C. antarctica</u> lipase A.

19. A DNA sequence encoding <u>C. antarctica</u> lipase A having the amino acid sequence shown in Fig. 1 or a modification of said DNA sequence which encodes a variant of <u>C. antarctica</u> lipase A which

1) has lipase activity,

2) reacts with an antibody reactive with at least one epitope of the <u>C. antarctica</u> lipase A having the amino acid sequence SEQ ID No. 1, and/or

3) is encoded by a nucleotide sequence which hybridizes with an oligonucleotide probe prepared on the basis of the full or partial nucleotide sequence shown in Fig. 1 encoding the <u>C. antarctica</u> lipase A.

20. A DNA construct comprising a DNA sequence encoding a lipase variant according to any of claims 1-17 or <u>C. antarc-</u>

<u>tica</u> lipase A according to claim 18.

21. A recombinant expression vector which carries a DNA construct according to claim 20.

22. A cell which is transformed with a DNA construct according to claim 20 or a vector according to claim 21.

23. A cell according to claim 22 which is a fungal cell, e.g. belonging to the genus <u>Aspergillus</u>, such as <u>A. niger</u>, <u>A.-oryzae</u>, or <u>A. nidulans</u>; a yeast cell, e.g. belonging to a strain of <u>Saccharomyces</u>, such as <u>S. cerevisiae</u>, or a methylotrophic yeast from the genera <u>Hansenula</u>, such as <u>H. polymorpha</u>, or <u>Phichia</u>, such as <u>P. pastoris</u>; or a bacterial cell, e.g. belonging to a strain of <u>Bacillus</u>, such as <u>B. subtilis</u>, or <u>B. lentus</u>, or to a strain of <u>Escherichia</u>, such as <u>E. coli</u>.

24. A method of producing a lipase variant according to any of claims 1-17, wherein a cell according to any of claims 21-23 is cultured under conditions conducive to the production of the lipase variant, and the lipase variant is subsequently recovered from the culture.

25. Use of a lipase variant according to any of claims 1-17 as an active ingredient in a detergent additive or a detergent composition.

26. Use of a lipase variant according to any of claims 1-17 or the <u>C. antarctica</u> lipase A or a variant thereof according to claim 18 in ester hydrolysis, ester synthesis or interesterification.

27. The use according to claim 260, in which the lipase variant is the lipase variant according to any of claims 9-11.

28. Use of a lipase variant according to any of claims 1-17

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or the C. antarctica lipase A or a variant thereof according to claim 18 for avoiding pitch trouble in a process for the production of mechanical pulp or a paper-making process using mechanical pulp.

5

29. The use according to claim 28, in which the lipase variant is the lipase variant according to any of claims 9-11.

C.antarctica lipase A.
Sequence of mature enzyme

AALPNPYDDPFYTTPSNIGTFAKGQVIQSRKVPTDIGNANNAASFQLQYRTTNTQNEAVADVATVW
IPAKPASPPKIFSYQVYEDATALDCAPSYSYLTGLDQPNKVTAVLDTPIIIGWALQQGYYVVSSDH
EGFKAAFIAGYEEGMAILDGIRALKNYQNLPSDSKVALEGYSGGAHATVWATSLAESYAPELNIVG
ASHGGTPVSAKDTFTFLNGGPFAGFALAGVSGLSLAHPDMESFIEARLNAKGQRTLKQIRGRGFCL
PQVVLTYPFLNVFSLVNDTNLLNEAPIASILKQETVVQAEASYTVSVPKFPRFIWHAIPDEIVPYQ
PAATYVKEQCAKGANINFSPYPIAEHLTAEIFGLVPSLWFIKQAFDGTTPKVICGTPIPAIAGITT
PSADQVLGSDLANQLRSLDGKQSAFGKPFGPITPP

Fig. 1A

C. antarctica lipase A.
Sequence of preproenzyme

```
MRVSLRSITSLLAAATAAVLAAPAAETLDRRAALPNPYDDPFYTTPSNIGTFAKGQVIQSRKVPTD
IGNANNAASFQLQYRTTNTQNEAVADVATVWIPAKPASPPKIFSYQVYEDATALDCAPSYSYLTGL
DQPNKVTAVLDTPIIIGWALQQGYYVVSSDHEGFKAAFIAGYEEGMAILDGIRALKNYQNLPSDSK
VALEGYSGGAHATVWATSLAESYAPELNIVGASHGGTPVSAKDTFTFLNGGPFAGFALAGVSGLSL
AHPDMESFIEARLNAKGQRTLKQIRGRGFCLPQVVLTYPFLNVFSLVNDTNLLNEAPIASILKQET
VVQAEASYTVSVPKFPRFIWHAIPDEIVPYQPAATYVKEQCAKGANINFSPYPIAEHLTAEIFGLV
PSLWFIKQAFDGTTPKVICGTPIPAIAGITTPSADQVLGSDLANQLRSLDGKQSAFGKPFGPITPP
```

Fig . 1B

Prepro C.antarctica lipase A -> 1-phase Translation

DNA sequence   1386 b.p.   ATGCGAGTGTCC ... ATCACACCACCT   linear

```
1/1                              31/11
ATG CGA GTG TCC TTG CGC TCC ATC ACG TCG CTG CTT GCG GCG GCA ACG GCG GCT GTG CTC
Met arg val ser leu arg ser ile thr ser leu leu ala ala ala thr ala ala val leu
61/21                            91/31
GCG GCT CCG GCG GCC GAG ACG CTG GAC CGA CGG GCG GCG CTG CCC AAC CCC TAC GAC GAT
ala ala pro ala ala glu thr leu asp arg arg ala ala leu pro asn pro tyr asp asp
121/41                           151/51
CCC TTC TAC ACG ACG CCA TCC AAC ATC GGC ACG TTT GCC AAG GGC CAG GTG ATC CAA TCT
pro phe tyr thr thr pro ser asn ile gly thr phe ala lys gly gln val ile gln ser
181/61                           211/71
CGC AAG GTG CCC ACG GAC ATC GGC AAC GCC AAC AAC GCT GCG TCG TTC CAG CTG CAG TAC
arg lys val pro thr asp ile gly asn ala asn asn ala ala ser phe gln leu gln tyr
241/81                           271/91
CGC ACC ACC AAT ACG CAG AAC GAG GCG GTG GCC GAC GTG GCC ACC GTG TGG ATC CCG GCC
arg thr thr asn thr gln asn glu ala val ala asp val ala thr val trp ile pro ala
301/101                          331/111
AAG CCC GCT TCG CCG CCC AAG ATC TTT TCG TAC CAG GTC TAC GAG GAT GCC ACG GCG CTC
lys pro ala ser pro pro lys ile phe ser tyr gln val tyr glu asp ala thr ala leu
361/121                          391/131
GAC TGT GCT CCG AGC TAC AGC TAC CTC ACT GGA TTG GAC CAG CCG AAC AAG GTG ACG GCG
asp cys ala pro ser tyr ser tyr leu thr gly leu asp gln pro asn lys val thr ala
421/141                          451/151
GTG CTC GAC ACG CCC ATC ATC ATC GGC TGG GCG CTG CAG CAG GGC TAC TAC GTC GTC TCG
val leu asp thr pro ile ile ile gly trp ala leu gln gln gly tyr tyr val val ser
481/161                          511/171
TCC GAC CAC GAA GGC TTC AAA GCC GCC TTC ATC GCT GGC TAC GAA GAG GGC ATG GCT ATC
ser asp his glu gly phe lys ala ala phe ile ala gly tyr glu glu gly met ala ile
541/181                          571/191
CTC GAC GGC ATC CGC GCG CTC AAG AAC TAC CAG AAC CTG CCA TCC GAC AGC AAG GTC GCT
leu asp gly ile arg ala leu lys asn tyr gln asn leu pro ser asp ser lys val ala
601/201                          631/211
CTT GAG GGC TAC AGT GGC GGA GCT CAC GCC ACC GTG TGG GCG ACT TCG CTT GCT GAA TCG
leu glu gly tyr ser gly gly ala his ala thr val trp ala thr ser leu ala glu ser
661/221                          691/231
TAC GCG CCC GAG CTC AAC ATT GTC GGT GCT TCG CAC GGC GGC ACG CCC GTG AGC GCC AAG
tyr ala pro glu leu asn ile val gly ala ser his gly gly thr pro val ser ala lys
721/241                          751/251
GAC ACC TTT ACA TTC CTC AAC GGC GGA CCC TTC GCC GGC TTT GCC CTG GCG GGT GTT TCG
asp thr phe thr phe leu asn gly gly pro phe ala gly phe ala leu ala gly val ser
781/261                          811/271
GGT CTC TCG CTC GCT CAT CCT GAT ATG GAG AGC TTC ATT GAG GCC CGA TTG AAC GCC AAG
gly leu ser leu ala his pro asp met glu ser phe ile glu ala arg leu asn ala lys
841/281                          871/291
GGT CAG CGG ACG CTC AAG CAG ATC CGC GGC CGT GGC TTC TGC CTG CCG CAG GTG GTG TTG
gly gln arg thr leu lys gln ile arg gly arg gly phe cys leu pro gln val val leu
901/301                          931/311
ACC TAC CCC TTC CTC AAC GTC TTC TCG CTG GTC AAC GAC ACG AAC CTG CTG AAT GAG GCG
thr tyr pro phe leu asn val phe ser leu val asn asp thr asn leu leu asn glu ala
961/321                          991/331
CCG ATC GCT AGC ATC CTC AAG CAG GAG ACT GTG GTC CAG GCC GAA GCG AGC TAC ACG GTA
pro ile ala ser ile leu lys gln glu thr val val gln ala glu ala ser tyr thr val
1021/341                         1051/351
TCG GTG CCC AAG TTC CCG CGC TTC ATC TGG CAT GCG ATC CCC GAC GAG ATC GTG CCG TAC
ser val pro lys phe pro arg phe ile trp his ala ile pro asp glu ile val pro tyr
1081/361                         1111/371
CAG CCT GCG GCT ACC TAC GTC AAG GAG CAA TGT GCC AAG GGC GCC AAC ATC AAT TTT TCG
gln pro ala ala thr tyr val lys glu gln cys ala lys gly ala asn ile asn phe ser
1141/381                         1171/391
CCC TAC CCG ATC GCC GAG CAC CTC ACC GCC GAG ATC TTT GGT CTG GTG CCT AGC CTG TGG
pro tyr pro ile ala glu his leu thr ala glu ile phe gly leu val pro ser leu trp
1201/401                         1231/411
TTT ATC AAG CAA GCC TTC GAC GGC ACC ACA CCC AAG GTG ATC TGC GGC ACT CCC ATC CCT
phe ile lys gln ala phe asp gly thr thr pro lys val ile cys gly thr pro ile pro
```

Fig 1C

```
1261/421                               1291/431
GCT ATC GCT GGC ATC ACC ACG CCC TCG GCG GAC CAA GTG CTG GGT TCG GAC CTG GCC AAC
ala ile ala gly ile thr thr pro ser ala asp gln val leu gly ser asp leu ala asn
1321/441                               1351/451
CAG CTG CGC AGC CTC GAC GGC AAG CAG AGT GCG TTC GGC AAG CCC TTT GGC CCC ATC ACA
gln leu arg ser leu asp gly lys gln ser ala phe gly lys pro phe gly pro ile thr
1381/461
CCA CCT
pro pro
```

Fig 1C (cont.)

```
                10        20        30        40        50        60   Res
                |         |         |         |         |         |
Rhizomucor
miehei
              ---------------------------SIDGGIRAATSQEINELTYYTTLSANSYCRTV   32
R. niveus    DDNLVGGMTLDLPSDAPPISLSSSTNSASDGGKVVAATTAQIQEFTKYAGIAATAYCRSV   60

                70        80        90       100       110       120   Res
                |         |         |         |         |         |
             IPGATWDCIHCDATE-DLKIIKTWSTLIYDTNAMVARGDSEKTIYIVFRGSSSIRNWIAD   91
             VPGNKWDCVQCQKWVPDGKIITTFTSLLSDTNGYVLRSDKQKTIYLVFRGTNSFRSAITD  120

               130       140       150       160       170       180   Res
                |         |         |         |         |         |
             LTFVPVSYPPVSGTKVHKGFLDSYGEVQNELVATVLDQFKQYPSYKVAVTGHSLGGATAL  151
             IVFNFSDYKPVKGAKVHAGFLSSYEQVVNDYFPVVQEQLTAHPTYKVIVTGHSLGGAQAL  180

               190       200       210       220       230       240   Res
                |         |         |         |         |         |
             LCALGLYQREEGLSSSNLFLYTQGQPRVGDPAFANYVVSTGIPYRRTVNERDIVPHLPPA  211
             LAGMDLYQREPRLSPKNLSIFTVGGPRVGNPTFAYYVESTGIPFQRTVHKRDIVPHVPPQ  240

               250       260       270       280       290       300   Res
                |         |         |         |         |         |
             AFGFLHAGEEYWITDNSPETVQVCTSDLETSDCSNSIVPFTSVLDHLSYFGINTGLCS   269
             SFGFLHPGVESWIKSGTSN-VQICTSEIETKDCSNSIVPFTSILDHLSYFDINEGSCL   297
```

Fig. 2

```
  1  MDDSVNTRYP  ILLVHGLFGF  DRIGSHHYFH  GIKQALNECG  ASVFVPIISA
 51  ANDNEARGDQ  LLKQIHNLRR  QVGAQRVNLI  GHSQGALTAR  YVAAIAPELI
101  ASVTSVSGPN  HGSELADRLR  LAFVPGRLGE  TVAAALTTSF  SAFLSALSGH
151  PRLPQNALNA  LNALTTDGVA  AFNRQYPQGL  PDRWGGMGPA  QVNAVHYYSW
201  SGIIKGSRLA  ESLNLLDPLH  NALRVFDSFP  TRETRENDGM  VGRFSSHLGQ
251  VIRSDYPLDH  LDTINHMARG  SAGASTR
```

Fig 3

```
                    X                    Y
         42        ▬▬▬             ▬▬▬▬        ? 89
A37916   TYGDEDCLYL NIWVPQGRK. ..QVSRDLPV MIWIYGGAFL MGSGHGANFL
 Lirtt   EVVTEDGYIL GVYRIPHGKN NSENIGKRPV VYLQHGLIAS AT..NWIANL
S07145   EVVTEDGYIL EVNRIPYGKK NSGNTGQRPV VFLQHGLLAS AT..NWISNL
  Lidg   TNKNPNNFQT LLPSDPSTIE ASNFQTDKKT RFTIHGFINK GE.ENWLLDM
A34494   TNENPNNFQE VA.ADSSSIS GSNFKTNRKT RFIIHGFIDK GE.ENWLANV
A34671   TNENPNNYQI ISATDPATIN ASNFQLDRKT RFIIHGFIDK GE.EGWLLDM
  Lipg   TNQNQNNYQE LV.ADPSTIT NSNFRMDRKT RFIIHGFIDK GE.EDWLSNI
A33553   GETNQ..GCQ IRINHPDTLQ ECGFNSSLPL VMIIHGWSVD GVLENWIWQM
A27442   KDESDRLGCQ LRPQHPETLQ ECGFNSSHPL VMIIHGWSVD GLLETWIWKI


         90                                          130
A37916   NNYLYDGEEI ATRGNVIVVT FNYRVGPLGF LSTGDANLPG NYGLRDQHMA
 Lirtt   PNNSLAFMLA DAGYDVWLGN SRGNTWSRKN VYYSPDSVEF WAFSFDEMAK
S07145   PNNSLAFILA DAGYDVWLGN SRGNTWARRN LYYSPDSVEF WAFSFDEMAK
  Lidg   CKNMFKVEE. ........VN CICVDWKKGS QTSYTQAANN VRVVGAQVAQ
A34494   CKNLFKVES. ........VN CICVDWKGGS RTGYTQASQN IRIVGAEVAY
A34671   CKKMFQVEK. ........VN CICVDWKRGS RTEYTQASYN TRVVGAEIAF
  Lipg   CKNLFKVES. ........VN CICVDWKGGS RTGYTQASQN IRIVGAEVAY
A33553   VAALKSQPAQ P.......VN VGLVDWITLA HDHYTIAVRN TRLVGKEVAA
A27442   VGALKSRQSQ P.......VN VGLVDWISLA YQHYAIAVRN TRVVGQEVAA


         131                                         175
A37916   IAWVKRNI.A AFGGDPNNIT │LFGESAGGAS│ VSLQTLSPYN K...GLIRRA
 Lirtt   YDLPATINFI VQKTGQEKIH │YVGHSQGTTI│ GFIAFSTNPT L..AKKIKTF
S07145   YDLPATIDFI VKKTGQKQLH │YVGHSQGTTI│ GFIAFSTNPS L..AKRIKTF
  Lidg   MLSMLS...A NYSYSPSQVQ │LIGHSLGAHV│ AGEAGSRTPG ...LGRITGL
A34494   FVEFLQ...S AFGYSPSNVH │VIGHSLGAHA│ AGEAGRRTNG T..IGRITGL
A34671   LVQVLS...T EMGYSPENVH │LIGHSLGSHV│ AGEAGRRLEG H..VGRITGL
  Lipg   FVEVLK...S SLGYSPSNVH │VIGHSLGSHA│ AGEAGRRTNG T..IERITGL
A33553   LLRWLE...E SVQLSRSHVH │LIGYSLGAHV│ SGFAGSSIGG THKIGRITGL
A27442   LLLWLE...E SMKFSRSKVH │LIGYSLGAHV│ SGFAGSSMGG KRKIGRITGL


         176                                         220
A37916   ISQSGVALSP WVIQKN.... ..PLFWAKKV AEKVGCPVGD AARMAQCLKV
 Lirtt   YALAPVATVK YTQSPLKKIS FIPTFLFKLM FGKKMFLPHT YFDDFLGTEV
S07145   YALAPVATVK YTKSLINKLR FVPQSLFKFI FGDKIFYPHN FFDQFLATEV
  Lidg   DPVEASFQGT PEEVRLD... ..PTDADFVD VIHTDAAPLI PFLGFGTSQQ
A34494   DPAEPCFQGT PELVRLD... ..PSDAKFVD VIHTDGAPIV PNLGFGMSQV
A34671   DPAEPCFQGL PEEVRLD... ..PSDAMFVD VIHTDSAPII PYLGFGMSQK
  Lipg   DPAEPCFQGT PELVRLD... ..PSDAKFVD VIHTDAAPII PNLGFGMSQT
A33553   DAAGPLFEGS APSNRLS... ..PDDASFVD AIHTFTREHM GLSVGIK.QP
A27442   DPAGPMFEGT SPNERLS... ..PDDANFVD AIHTFTREHM GLSVGIK.QP


                                 z = FLAP region
                              ▬▬▬▬▬▬▬▬▬▬▬▬▬▬
1─   A37916   TDPRALTLAY KVPLAGLEYP MLHYVGFVPV IDGDFIPADP INLYANAADI
2─   Lirtt    CSREVLDLLC SNTLFIFCGF DKKNLNVSRF DVYLGHNPAG TSVQDFLHWA
3─   S07145   CSREMLNLLC SNALFIICGF DSKNFNTSRL DVYLSHNPAG TSVQNMFHWT
4─   Lidg     MGHLDFFPNG GEEMPGCKKN ALSQIVNLDG IWEGTRDFVA CNHLRSYKYY
5─   A34494   VGHLDFFPNG GVEMPGCKKN ILSQIVDIDG IWEGTRDFAA CNHLRSYKYY
6─   A34671   VGHLDFFPNG GKEIPGCQKN ILSTIVDING IWEGTRNFAA CNHLRSYKYY
7─   Lipg     VGHLDFFPNG GKQMPGCQKN ILSQIVDIDG IWEGTRDFVA CNHLRSYKYY
8─   A33553   IGHYDFYPNG GSFQPGCHFL ELYRHIAQHG FNAITQTIK. CSHERSVHLF
9─   A27442   IAHYDFYPNG GSFQPGCHFL ELYKHIAEHG LNAITQTIK. CAHERSVHLF
```

Fig. 4

A A L P N P Y D D P F Y T T P S N

N-terminal sequence of C.antartica LF 058 lipase component A

; Guess positions indicated

```
AACCCATACGACGACCC        NOR 440
    T C T T T
        G
        T

GCTGCTCTGCCTAACCCTTACGACGACCCTTCTACACCACCCC        NOR 438
              T T C
```

Fig. 5

```
   1    TCGAACGATG CGAGTGTCCT TGCGCTCCAT CACGTCGCTG CTTGCGGCGG
  51    CAACGGCGGC TGTGCTCGCG GCTCCGGCGG CCGAGACGCT GGACCGACGG
 101    GCGGCGCTGC CCAACCCCTA CGACGATCCC TTCTACACGA CGCCATCCAA
 151    CATCGGCACG TTTGCCAAGG GCCAGGTGAT CCAATCTCGC AAGGTGCCCA
 201    CGGACATCGG CAACGCCAAC AACGCTGCGT CGTTCCAGCT GCAGTACCGC
 251    ACCACCAATA CGCAGAACGA GGCGGTGGCC GACGTGGCCA CCGTGTGGAT
 301    CCCGGCCAAG CCCGCTTCGC CGCCCAAGAT CTTTTCGTAC CAGGTCTACG
 351    AGGATGCCAC GGCGCTCGAC TGTGCTCCGA GCTACAGCTA CCTCACTGGA
 401    TTGGACCAGC CGAACAAGGT GACGGCGGTG CTCGACACGC CCATCATCAT
 451    CGGCTGGGCG CTGCAGCAGG GCTACTACGT CGTCTCGTCC GACCACGAAG
 501    GCTTCAAAGC CGCCTTCATC GCTGGCTACG AAGAGGGCAT GGCTATCCTC
 551    GACGGCATCC GCGCGCTCAA GAACTACCAG AACCTGCCAT CCGACAGCAA
 601    GGTCGCTCTT GAGGGCTACA GTGGCGGAGC TCACGCCACC GTGTGGGCGA
 651    CTTCGCTTGC TGAATCGTAC GCGCCCGAGC TCAACATTGT CGGTGCTTCG
 701    CACGGCGGCA CGCCCGTGAG CGCCAAGGAC ACCTTTACAT TCCTCAACGG
 751    CGGACCCTTC GCCGGCTTTG CCCTGGCGGG TGTTTCGGGT CTCTCGCTCG
 801    CTCATCCTGA TATGGAGAGC TTCATTGAGG CCCGATTGAA CGCCAAGGGT
 851    CAGCGGACGC TCAAGCAGAT CCGCGGCCGT GGCTTCTGCC TGCCGCAGGT
 901    GGTGTTGACC TACCCCTTCC TCAACGTCTT CTCGCTGGTC AACGACACGA
 951    ACCTGCTGAA TGAGGCGCCG ATCGCTAGCA TCCTCAAGCA GGAGACTGTG
1001    GTCCAGGCCG AAGCGAGCTA CACGGTATCG GTGCCCAAGT TCCCGCGCTT
1051    CATCTGGCAT GCGATCCCCG ACGAGATCGT GCCGTACCAG CCTGCGGCTA
1101    CCTACGTCAA GGAGCAATGT GCCAAGGGCG CCAACATCAA TTTTTCGCCC
1151    TACCCGATCG CCGAGCACCT CACCGCCGAG ATCTTTGGTC TGGTGCCTAG
1201    CCTGTGGTTT ATCAAGCAAG CCTTCGACGG CACCACACCC AAGGTGATCT
1251    GCGGCACTCC CATCCCTGCT ATCGCTGGCA TCACCACGCC CTCGGCGGAC
1301    CAAGTGCTGG GTTCGGACCT GGCCAACCAG CTGCGCAGCC TCGACGGCAA
1351    GCAGAGTGCG TTCGGCAAGC CCTTTGGCCC CATCACACCA CCTTAGTCGG
1401    CGAGCACTTT CGGATGTTCC GCTACAGTTC CCACGGTCGA TGCCGACCTT
1451    CAGAGATCAC GAGTGACCTC GGG
```

Fig 6

Table I

Amino acid composition of C. antartica lipase A (CALIP)

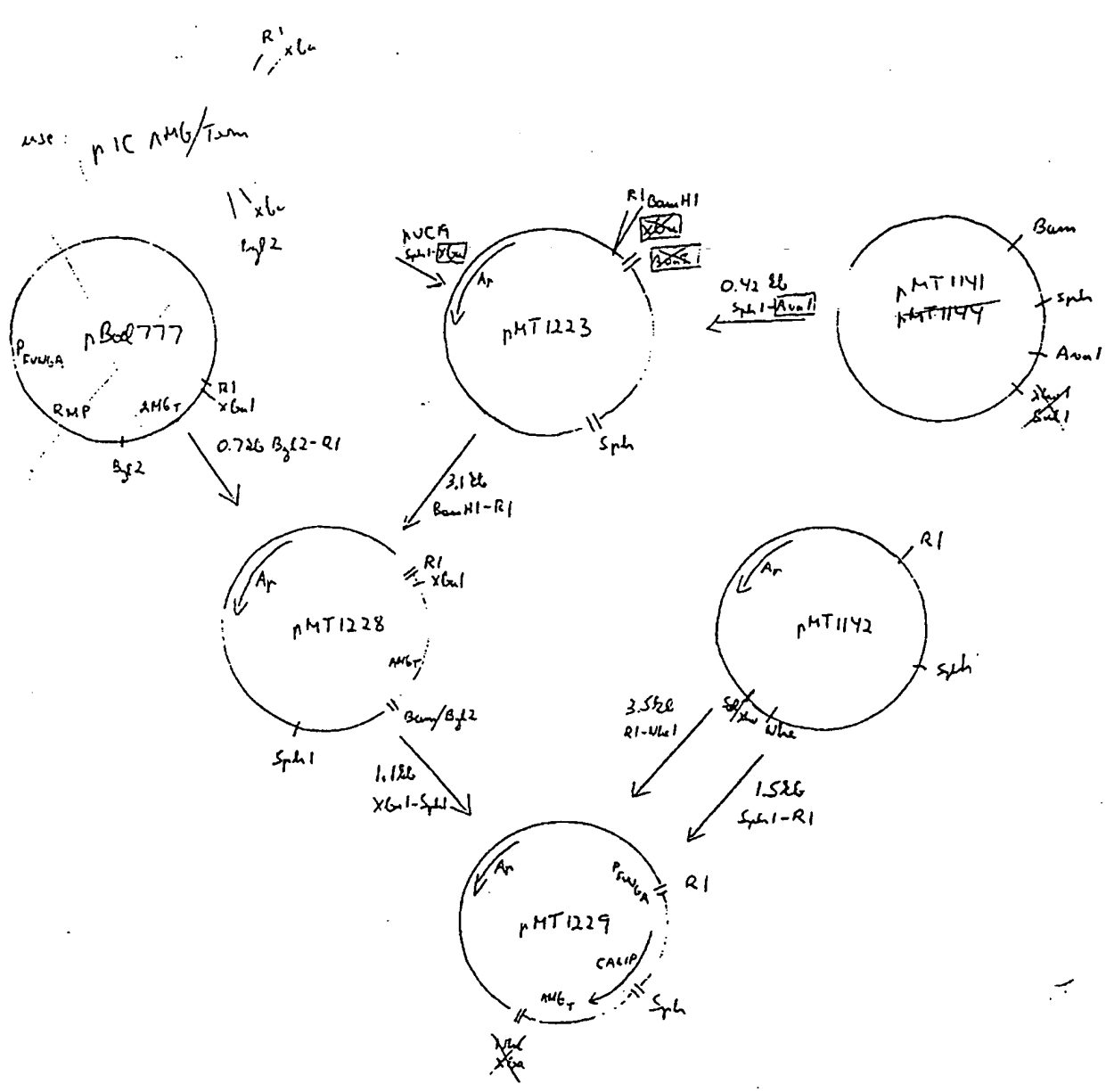|	Deduced from DNA sequence	By amino acid analysis (MC)
Ala	50	47
Arg	9	9
Asp/AsN	35	36
Cys	4	4
Gln/GlN	35	36
Gly	28	31
His	6	6
Ile	26	24
Leu	29	30
Lys	17	17
Met	2	3
Phe	20	19
Pro	33	33
Ser	26	27
Thr	27	28
Trp	5	4
Tyr	18	16
Val	27	26

Fig. 7

Fig. 8

Fig 8 : Construction of expression plasmid for Candutica lipase A